Directly Observed ^{15}N NMR Spectra of Uniformly Enriched Proteins†

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ABSTRACT: The proteins cytochrome c_2, cytochrome c', and ribulosebisphosphate carboxylase/oxygenase from *Rhodospirillum rubrum* were enriched in ^{15}N by growth of the organism on $^{15}NH_4Cl$. The proteins were purified to homogeneity and studied by ^{15}N NMR. Longitudinal and transverse relaxation times as well as the nuclear Overhauser effects were determined for various groups of the proteins which vary in molecular weight from 13 000 to 114 000. The values of these parameters for the amide resonances or for groups thought to be rigid were consistent with the molecular weights of the proteins. Relaxation times of the amino-terminal α-amino groups and the side chain nitrogen atoms of arginine and lysine were consistent with much more rapid motion. Nitrogen atoms having bound protons were generally found to be decoupled from the protons by chemical exchange. Demonstrable ^1H–^{15}N coupling was taken as an indication that exchange was hindered, either by hydrogen bonding interactions or by inaccessibility of the group to solvent. Histidine side chain nitrogen atoms, which experience a large chemical shift upon protonation/deprotonation, were often found to be broadened beyond detectability by chemical exchange and tautomerization. Strategies for improving sensitivity and for obtaining specific peak assignments are also discussed.

^{15}N is a spin $^1/_2$ nucleus with a natural abundance of 0.37%. Because of the unusually large response of its NMR chemical shift to changes in protonation state, hydrogen bonding, and metal ligation (Roberts, 1980; Kanamori & Roberts, 1983; Mason, 1981), it is recognized to have great potential as a "reporter group" for measuring these interactions in small molecules, proteins, and nucleic acids [e.g., see Sogn et al. (1973), Morishima and Inubushi (1977), and Griffey et al. (1983)]. This potential has historically not been fully realized because ^{15}N, in addition to its meager natural abundance, has a small negative magnetogyric ratio (γ) that is −0.1013 times that of a proton. NMR sensitivity, which depends on γ^3, is thus only 10^{-3} that of protons for the same number of spins. The ^{15}N signal at natural abundance is therefore about 4×10^{-6} that of protons at the same concentration.

There are several approaches that might be used to overcome this serious lack of sensitivity, including insensitive nucleus enhancement techniques employing J coupling (Morris & Freeman, 1979) or dipolar coupling (McArthur et al., 1969), proton-observed heteronuclear pulse sequences (Griffey et al., 1984), or simple isotopic enrichment. The applicability of each of these approaches is limited by various factors. For instance, the INEPT pulse sequence is restricted to systems in which $T_2 \gtrsim 1/J$, and cross-polarization is only useful where motion is relatively slow. Bachovchin and Roberts (1978) have used selective enrichment of the single histidyl residue of α-lytic protease to observe the effect of pH variations of its chemical shift. Their elegant approach entailed the use of a histidine-requiring auxotroph of myxobacter 495 and ^{15}N-labeled histidine and required the knowledge that the histidine was indeed the critical residue upon which mechanistic arguments could be built. If it had been necessary to observe another residue, e.g., tryptophan, a second sample, produced by another auxotroph from isotopically labeled tryptophan, would have been required. A less elegant though perhaps more general approach would be to use an organism capable of growth on ^{15}N-labeled ammonium ion as the sole nitrogen source to produce uniformly labeled proteins, nucleic acids, etc. Unlike carbon, nitrogen atoms are rarely bonded to each other in biomolecules so that there is no danger of losing signal intensity due to nitrogen–nitrogen coupling. Enrichment levels of >90% are thus both practical and attainable, providing the organism does not require significant amounts of compounds that could also be used as nitrogen sources. Although assignment ambiguities not encountered by Bachovchin and Roberts would be created, for some applications uniform enrichment might be both more economical and more generally useful.

We have obtained the proteins cytochrome c_2, cytochrome c', and ribulose-1,5-bisphosphate carboxylase (RuBPCase)[1] from the photosynthetic bacterium *Rhodospirillum rubrum* that were uniformly enriched in ^{15}N by growth on labeled ammonium ion. Their ^{15}N NMR spectra, relaxation times, and nuclear Overhauser effects (NOE's) were measured at 50.67 MHz. Our results are reported here together with an assessment of the usefulness of directly observed ^{15}N NMR spectroscopy of nonselectively enriched proteins as a tool for the study of protein structure and dynamics.

Materials and Methods

For isolation of the cytochromes, the G-9 mutant of *Rhodospirillum rubrum* was grown photosynthetically on malate medium (Ormerod et al., 1961) with biotin supplied as the only vitamin and 0.5 g/L $^{15}NH_4Cl$ (MSD Isotopes) as the nitrogen source. Growth was nitrogen limited at this level, and essentially no ^{15}N remained in the spent medium. The cytochromes were prepared from an EDTA wash of the cells as described by Bartsch (1978). They were found by polyacrylamide gel electrophoresis and by purity index to be essentially homogeneous.

To prepare RuBPCase, the same organism was grown photolithotrophically on 1.5% CO_2 in H_2 as the carbon source as described by Schloss et al. (1979) for several transfers to

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[1] Abbreviations: RuBPCase, ribulose-1,5-bisphosphate carboxylase/oxygenase; NOE, nuclear Overhauser effect; TEMMB, 50 mM tris(hydroxymethyl)aminomethane, 1 mM EDTA, 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and 50 mM $NaHCO_3$, pH 8.3; EDTA, ethylenediaminetetraacetate; MES, 2-(*N*-morpholino)ethanesulfonic acid; COSY, shift correlation spectroscopy; Tris, tris(hydroxymethyl)aminomethane.

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prepare a derepressed inoculum that was used to inoculate ^{15}N-enriched cultures in the same medium. Growth under these conditions was both slow and light, but the RuBPCase in the crude extracts of the derepressed cells was found to be >40% pure, so that 100-mg quantities of the enzyme could be prepared even from small quantities of cells by a minor modification of the method described by Schloss et al. (1979). The procedure involved ion-exchange chromatography on DEAE-cellulose equilibrated with 50 mM Tris buffer containing 50 mM $NaHCO_3$, 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and 1 mM EDTA, pH 8.3 (TEMMB), followed by precipitation by ammonium sulfate and gel filtration on Sephacryl S-200 equilibrated with 50 mM MES buffer containing 50 mM $NaHCO_3$, 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and 1 mM EDTA, pH 6.2, and chromatography on DEAE-cellulose in the second buffer. NaCl gradients of 0–200 mM were used in both ion-exchange steps. Phenylmethanesulfonyl fluoride (0.2 mM) was included in the initial extract to protect against proteolysis. Some preparations were resubmitted to the gel filtration step to improve purity. The RuBPCase was found to be >99% pure by polyacrylamide gel electrophoresis and had a correspondingly high specific activity (2.8 μmol of RuBP cleaved min^{-1}) when assayed by the method of Anderson (1975).

The cytochromes were concentrated by lyophilization; the RuBPCase was concentrated by ultrafiltration in a collodion bag (Schleicher & Schuell). Fifteen percent ^2H_2O was included in all buffers used for NMR as a lock compound. Parameters used for collecting and processing NMR spectra, including sample concentrations, are contained in the figure legends. The term "acquisition time" is used to mean the time during which data are actually being digitized and is distinguished from "recycle time" which is used to represent the total time between successive repetitions of the pulse sequence.

NMR spectra were collected at 50.67 MHz by using a General Electric NM-500 NMR spectrometer console with a micro-bore 11.7-T magnet from Oxford Instruments. Our wide-band probe which accommodates 10-mm NMR sample tubes was obtained from Cryomagnet Systems, Inc. Chemical shifts are referenced to 1 N HNO_3 in ^2H_2O, although they were generally measured from external or internal ammonium ion which was assigned a shift of 354.5 ppm. The spectra were routinely acquired by using a 8-kHz spectral window and an acquisition time of 0.5 s. NOE values are reported as a ratio of peak intensities in the presence and absence of the NOE (i.e., $1 + \epsilon$) rather than as the enhancement alone. T_1 measurements were made by the inversion-recovery method (Vold et al., 1968) using a composite 180° pulse. T_2 values were determined by using the spin-echo method (Hahn, 1950) as modified by Carr and Purcell (1954).

Theoretical values of relaxation times were calculated from the standard equations (Abragam, 1961) as were NOE's (Bothner-by & Gassend, 1973). Rotational correlation times for proteins were calculated from their molecular weights as described by Woessner (1962).

Results and Discussion

Chemical Shifts. The proton-coupled ^{15}N NMR spectra of cytochrome c_2, cytochrome c', and RuBPCase are shown in Figure 1. Nitrogen chemical shifts have been reviewed most recently by Witanowski et al. (1981). There are clearly four distinct categories of chemical shifts: the amine region (325–350 ppm), containing resonances of the ϵ-amino nitrogen of lysine and free α-amino groups; the guanidino nitrogen region (290–310 ppm), containing the resonances of both N^{δ} and N^{ω} nitrogens of arginine; the amide region, containing the resonance of peptide nitrogens (240–260 ppm) and other amides (260–275 ppm); and the "aromatic" nitrogen region (downfield of 250 ppm), containing the resonances of tryptophan and histidine and heme ring nitrogen atoms. It is also clear that the resonances of individual nitrogen atoms are clearly resolved in general only when they arise from a relatively rare residue or when they have some unusual interaction that distinguishes their chemical environment from those of other residues of the same type. The resonances of lysyl ϵ-amino groups or the guanidino nitrogens of arginine form a relatively featureless envelope, probably because they are usually on the surface of the protein and experience similar environments.

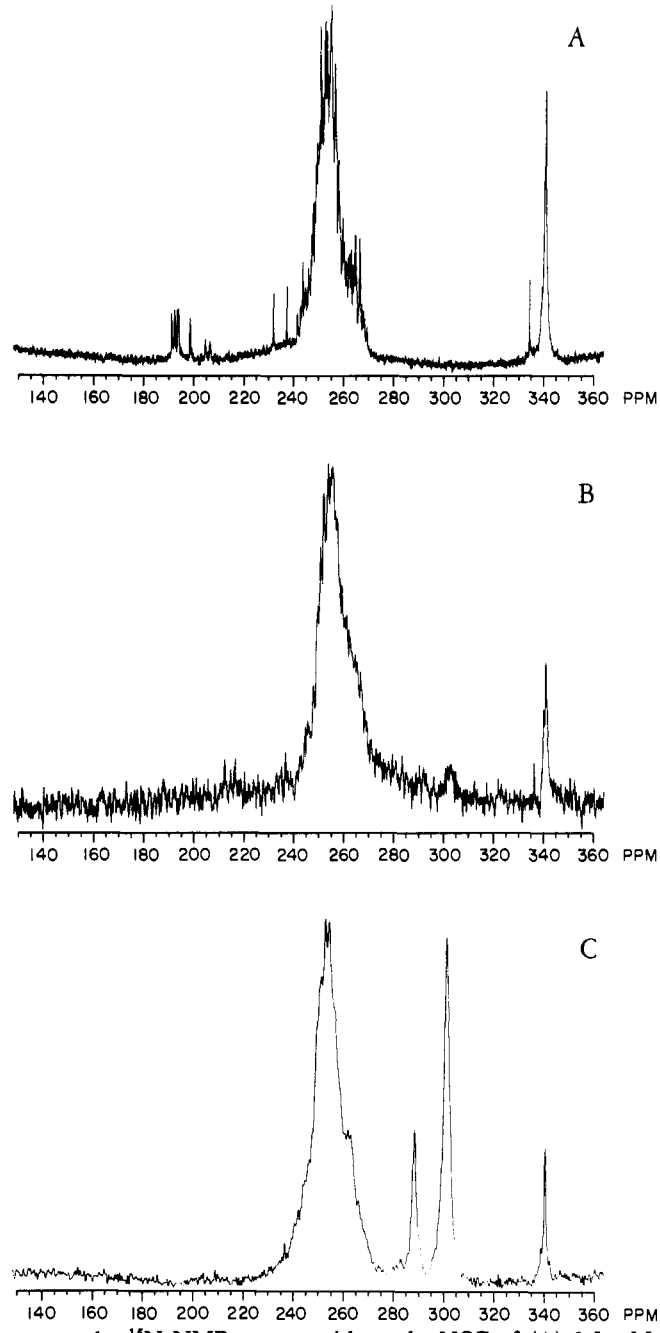

FIGURE 1: ^{15}N NMR spectra without the NOE of (A) 3.5 mM cytochrome c_2, pH 5.5, (B) 1.8 mM cytochrome c' (based on heme), pH 6.5, and (C) 2 mM RuBPCase dimer at 50.67 MHz in 15% ^2H_2O. Instrumental parameters are as follows: (A) 3072 scans, 5-s recycle time; (B) 1024 scans, 1-s recycle time; (C) 4096 scans, 10-s recycle time.

Nuclear Overhauser Effects. Proton-coupled ^{15}N NMR spectra of cytochrome c_2, cytochrome c', and RuBPCase ob-

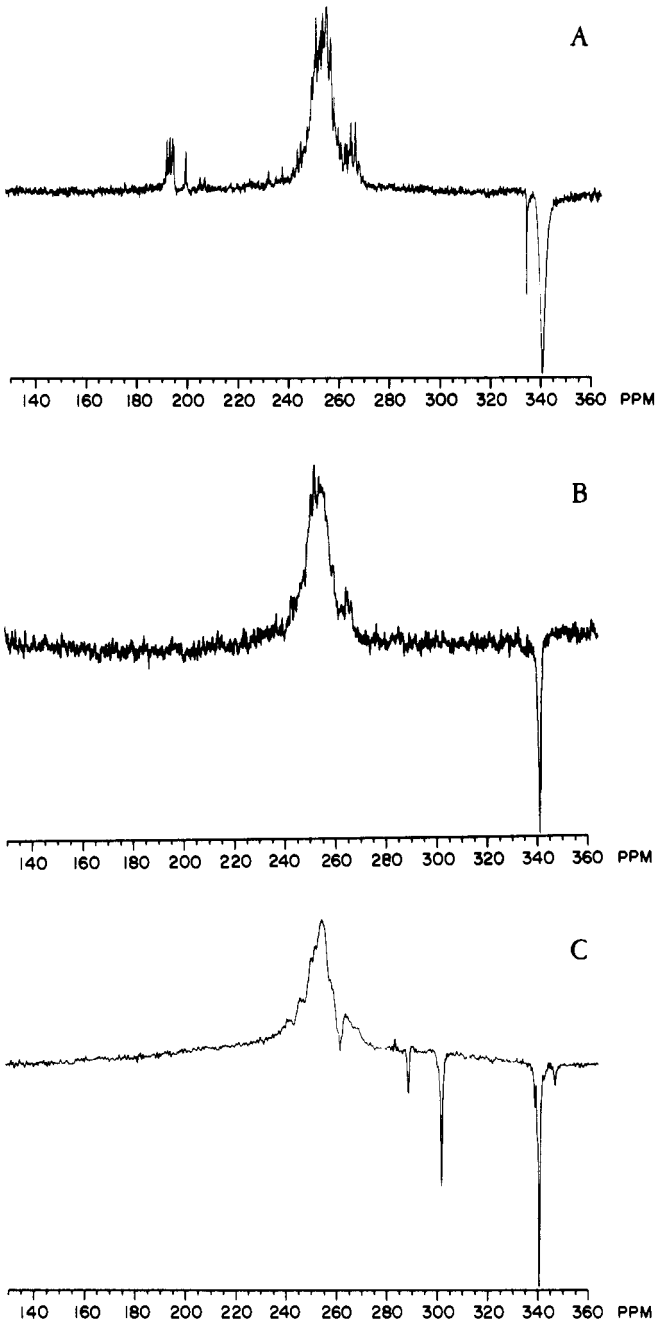

FIGURE 2: ^{15}N NMR spectra with the NOE of (A) cytochrome c_2, (B) cytochrome c', and (C) RuBPCase at 50.67 MHz. Parameters are the same as in Figure 1 except in (A) where 1024 scans and 1-s recycle times were used.

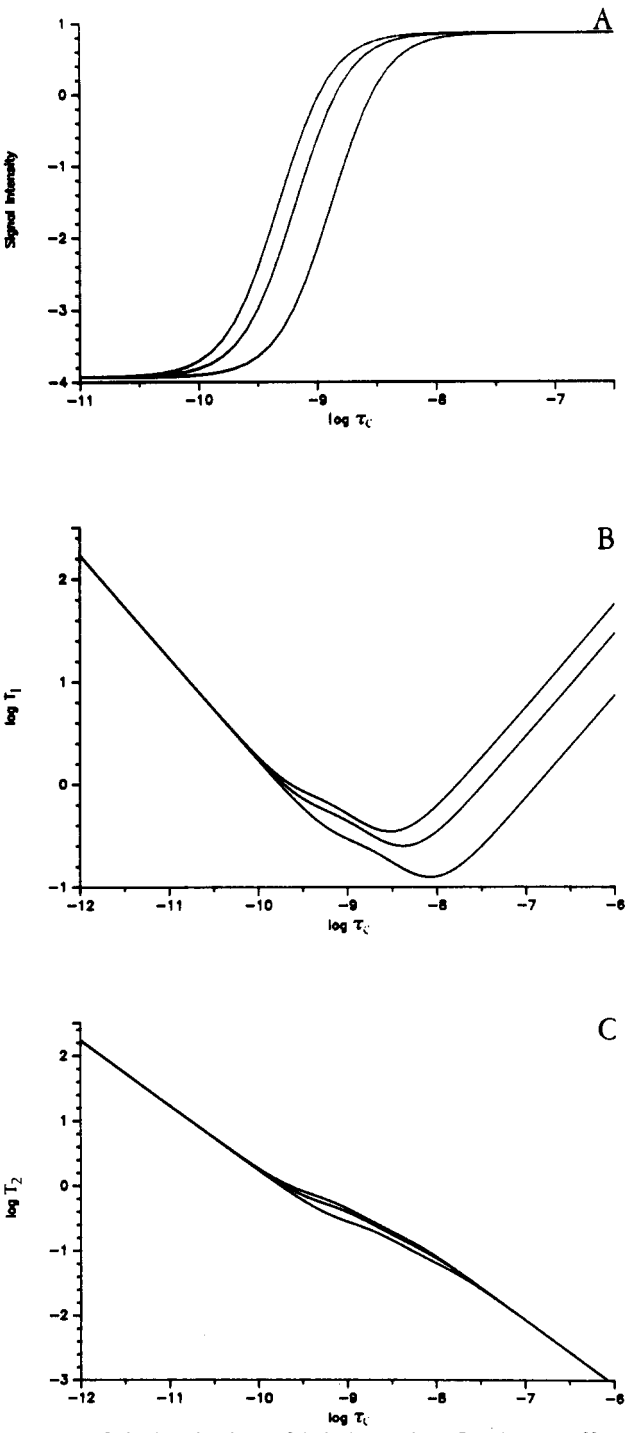

FIGURE 3: Calculated values of (A) the nuclear Overhauser effect, (B) T_1, and (C) T_2 of ^{15}N as a function of rotational correlation time. The dipole–dipole interaction between nitrogen and a single proton at a distance of 1 Å, modulated by isotropic reorientation, is assumed to provide the only mechanism of relaxation. The calculations were carried out for nitrogen resonance frequencies of 18.25, 36.51, and 50.67 MHz, increasing from bottom to top in the figures. Relaxation times are expressed in seconds.

tained with broad-band irradiation of protons during the delay are shown in Figure 2. Because of the negative γ value of ^{15}N, the nuclear Overhauser effect causes inversion of some of the peaks. The sign and magnitude of the NOE depend on the correlation time, τ_c, of the motion modulating the dipole–dipole interaction that leads to longitudinal relaxation. The dependence on τ_c of the NOE, calculated for isotropic reorientation, is shown in Figure 3. Most proteins are expected to have overall tumbling times of greater than 1 ns. Thus, resonances arising from nitrogens that are rigidly fixed within the protein are expected to exhibit a slight decrease in signal intensity if they receive an NOE; the limiting value for intensity at large values of τ_c is 0.877. Small molecules show signals −3.93 times that obtained in the absence of the NOE. Groups in the protein that are mobile due to segmental, side chain, or rotational motion may exhibit NOE values characteristic of much smaller molecules. The NOE values found for groups having different apparent motional freedom are given in Table I.

With the exception of the heme resonances of cytochrome c_2, the NOE's found for groups known to be governed by the overall tumbling of the protein are indeed consistent with the correlation time calculated from the Stokes equation; those calculated from bulk or unresolved resonances appear to be slightly smaller owing to the presence of more mobile groups

Table I: Relaxation Times and NOE's of ^{15}N Resonances of Ammonium Ion, Cytochrome c_2, Cytochrome c', and RuBPCase

				rigid or bulk			mobile[a]			
sample	M_r	τ_c[b] (ns)		T_1 (s)	T_2 (s)	NOE (s)	T_1 (s)	T_2 (s)	NOE (s)	
ammonium	19	0.003					52	0.03	−3.9	
cyt c_2	13000	4	peptide	0.52	0.03	0.77	2.3	0.02	−2.8	Lys N^ϵ
			heme	0.2	0.03[c]	~1	1.1	0.14	−2.7	N-terminus
cyt c'	28000	8	peptide	0.89	0.02	0.77	2.0	0.04	−2.2	Lys N^ϵ
RuBPCase	114000	33	peptide	2.6	0.009	0.6	1.7	0.02	−0.3	Arg N^δ
							0.86	0.005	−0.4	Arg N^ω
							1.30	0.04	−3.2	Lys N^ϵ

[a] The distinction between mobile and rigid groups is based solely on the sign of the NOE. [b] Rotational correlation times for proteins were calculated by assuming spherical molecules of partial specific volume 0.725, at T = 298 K and solvent viscosity = 1.022 cP. The effects of hydration, which may be significant, are ignored. The value of τ_c for ammonium was calculated from $\tau_c = 4\pi\eta r^3/kT$ with r = 1 Å. The relaxation data were obtained from the samples used for Figures 1 and 2. [c] Determined from line width.

resonating under the same envelope. The peaks assigned to the heme of the ferrocytochrome receive almost no NOE. This result is not surprising; the pyrrole nitrogens have no bound and few nearby protons, and even if they received a full NOE, their intensities would only be reduced slightly to ~0.9. The contribution of other relaxation mechanisms such as shift anisotropy to relaxation rates of the heme may also reduce the observed NOE. Field-dependence data on the relaxation times, which are not yet available, would allow assessment of the contribution of this mechanism to the overall relaxation times.

Lysines and arginines have considerable side chain motion, leading to strongly negative peaks which were actually observed by Gust et al. (1975) at natural abundance. One possible exception is the single Arg residue of cytochrome c' which appears to have an NOE of about 0. However, the breadth of the peak in the absence of the NOE suggests that it is close to the paramagnetic center which, by contributing to longitudinal relaxation, could reduce the magnitude of the NOE. The mobility of the N-terminus appears to vary. The N-terminal glutamate in cytochrome c_2, which is at the end of a surface α-helix, appears to be quite mobile, receiving an NOE of −2.7; the N-terminus of cytochrome c' appears less so, receiving an NOE of ~0. The resonance of the N-terminus of RuBPCase is visible only with the NOE. The conclusion that the negative NOE is a result of local motion must be drawn with caution, however. Since most nitrogen atoms have exchangeable attached protons, it is possible that in some cases proton exchange might be the process that modulates the dipolar interaction. In these cases, it would be the rapid variation in internuclear distance rather than the variation in the direction of the internuclear vector whose correlation time controls relaxation and the NOE.

We have found that the large negative NOE experienced by lysine ϵ-amino groups is actually very difficult to suppress by the usual gated decoupling techniques. The NOE is expected to be governed by the saturation produced by the irradiation of bound protons, which should have reasonably short T_1 values. However, the protons of H_2O which have relatively long T_1's become saturated easily by the decoupler during the acquisition time and produce the NOE by chemical exchange with the amino protons. It is often necessary to employ short acquisition times and long recycle times to remove the NOE.

An additional caveat concerning the NOE should be noted. At the ^{1}H frequency employed in this study (500 MHz) and especially at relatively long correlation times, the phenomenon of spin diffusion (Kalk & Berendsen, 1976) is expected to be significant. This effect seriously limits the selectivity of proton NOE's (Bothner-By & Gassend, 1973) because the energy-conserved zero-quantum transition ("cross relaxation") dominates longitudinal relaxation at long τ_c and allows energy to be passed relatively efficiently from spin to spin, throughout the protein. The selectivity of ^{1}H–^{15}N would also be destroyed if an individual proton resonance cannot be saturated selectively. We have observed irradiation in the aliphatic region of the ^{1}H spectrum leading to NOE's in the lysine region of the ^{15}N spectrum even in the smallest of the three proteins under study.

Relaxation Times and Line Widths. Similar to carbon, ^{15}N relaxes primarily by the dipolar interaction with attached protons. For isotropically reorienting molecules, the frequency and τ_c dependence of dipolar relaxation times can be calculated easily (Abragam, 1961); the absolute relaxation times depend on the number of interacting protons and their distances from the nucleus. The relaxation times calculated by assuming that one attached proton at a distance of 1.0 Å dominates the relaxation, as might be expected for peptide groups, are shown in Figure 3. Although there is an ambiguity in the relationship between τ_c and T_1 because a particular T_1 value could arise from either of two τ_c's the availability of NOE data allows the distinction to be made. The apparent (or aggregate) T_1 values obtained for the peptide resonances agree roughly with the calculated values of τ_c: correlation times of 1, 16, and 45 ns are obtained, respectively, for cytochromes c_2 and c' and for RuBPCase, assuming relaxation primarily by a single proton at a distance of 1 Å. These values may be compared with those calculated from the Stokes equation which are listed in Table I. The excessively high value for cytochrome c' could result from the formation of higher oligomers at the high concentration used for the T_1 measurements. The effect of the high-spin Fe(III) on the bulk relaxation times appears to be negligible because it would decrease the observed T_1, making τ_c appear anomalously short rather than long. The only effect of the unpaired electron of cytochrome c' appears to be to broaden the resonance of the single Arg in the sequence.

As in the case of the NOE's, groups that have their own (greater) motional freedom exhibit relaxation times that are characteristic of shorter correlation times. The guanidino nitrogens of RuBPCase are a particularly good example; their T_1 values differ by a factor of 2, the δ-nitrogen having one attached proton, the ω-nitrogens each having two. Therefore, they must have the same correlation time which, being much shorter than the overall tumbling time as evidenced by the negative NOE, must arise from rotation about the C^γ–N^δ bond or an adjacent bond in the side chain. Anisotropic motion such as rotation about a bond axis must be accounted for by a second correlation time. Because the relaxation fit a single exponential, it is assumed that cross-correlation effects between the two motions are small (Werbelow & Marshall, 1973; Coates et al., 1973). The relaxation can be described in terms of the correlation time for rotation about the bond axis (τ_r) and the reorientation of the bond axis itself (presumed to equal

overall tumbling, τ_c, as a first approximation) which combine to form a composite correlation time, $(1/\tau_c + 1/\tau_r)^{-1}$. As in the case of the rotation of a methyl group, the relaxation equation contains terms in both τ_c and the composite correlation time. If the rotation axes reorient with a correlation time of ~40 ns, the overall tumbling time, the correlation time for the rotation about the bond can be calculated to be $\sim 10^{-11}$ s. The motions could be determined more reliably if frequency dependence data on both T_1 and the NOE were available (Jardetzky, 1981).

For correlation times greater than $1/\omega$, T_2 is expected to be considerably shorter than the T_1 of the same spin as is observed. However, for groups with shorter correlation times, the observed T_2 values do not appear to agree with theory as well as those obtained for T_1. Indeed, the T_2 of ammonium, which should be in the "extreme narrowing" region where $T_2 = T_1$, is 3 orders of magnitude shorter than the T_1. The Carr–Purcel–Meiboom–Gill method for the determination should remove the contribution of field inhomogeneity and minimize the effects of molecular diffusion within the sample tube. It is thus reasonable to conclude that other relaxation mechanisms, specifically scalar relaxation caused by proton exchange modulation of J coupling, are operative. This observation points out the inadequacy of using T_1:T_2 ratios to determine correlation times.

The histidine nitrogens of RuBPCase, of which there are 10, did not produce sharp signals. This observation also is attributed to chemical exchange between the two proton tautomers. The chemical shift difference between imidazole-type and imidazolium-type nitrogens is about 20–60 ppm (1–3 kHz). Chemical exchange that is fast compared to the proton shift difference (~1 ppm or ~200–500 Hz) may be only intermediate compared to the nitrogen shift difference. The resonances may therefore broaden severely. The spectra were acquired in and "activating buffer" (TEMMB) which contained magnesium ion to preserve enzymatic activity during the experiment. The requirement for the metal ion made it impossible to treat the buffer with chelating resins or to remove paramagnetic metal contaminants by other standard procedures. Therefore, paramagnetic ions could be present that bind to the His nitrogen atoms to cause broadening. The buffer contained EDTA which, although at low concentration, has several orders of magnitude higher affinity for Mn^{2+}, Fe^{2+}, Fe^{3+}, and Cu^{2+} and other likely contaminants that for Mg^{2+}, and thus should bind them preferentially. Bachovchin and Roberts (1978) also found that the N^{τ} of [^{15}N]His α-lytic protease was not visible at intermediate pH values, suggesting the broadening of histidine resonances to be a general phenomenon.

Another result of proton exchange is that it tends to cause decoupling of the ^{15}N NMR spectrum of nitrogens with exchangeable protons. A typical one-bond coupling constant is ~90 Hz; thus, even a modest exchange rate of ~100 s^{-1} is sufficient to effect decoupling. Indeed, only in cases in which exchangeable protons of histidine or tryptophan were thought to be hydrogen bonded did we observe splitting of the ^{15}N NMR line (see, for instance, the doublet at 205 ppm in the spectrum of cytochrome c_2). In general, the observation of proton–nitrogen coupling indicates that proton exchange is considerably slower than 100 s^{-1}. The side chain N–H protons of histidine, arginine, and lysine are expected to exchange more rapidly if they are exposed to solvent. Therefore, the observation for proton–nitrogen coupling is excellent evidence either that the couple is sequestered away from the solvent or that exchange is hindered by hydrogen bonding. Exchange that is fast enough to produce decoupling also leads to scalar relaxation, as described above.

Except for that in aromatic side chains, nitrogen–proton coupling through more than one bond is only a few hertz and does not result in resolved splitting of either the proton or the nitrogen resonance. In histidine and tryptophan, three-bond coupling is expected to be ~12 Hz and may be useful in obtaining assignments.

Strategies for Assignment. Except for the distinction between the amide nitrogens of glutamine and asparagine, the chemical shifts of all side chain nitrogen atoms of the common amino acids are sufficiently different to allow the assignment of a resonance to a particular amino acid type. The next most useful information concerns proton coupling. In comparison to ^{13}C, this information is less readily obtainable because directly bound protons are generally exchangeable. We have found selective proton decoupling experiments to be disappointing for this season. In addition, heteronuclear shift correlation experiments are difficult because they require a "mixing time" on the order of $1/2J$. For ^{15}N in proteins, this value may be similar to or longer than T_2, so that the magnetization decays before the effect is complete. For carbon, mixing times of $1/4J$ are appropriate for methylene and methyl carbons (i.e., triplets and quartets) with $0.3/J$ often taken as a compromise (Bax, 1984). However, most nitrogens of interest are N–H groups (nitrogen doublets); the magnetization that is modulated in the t^1 dimension is largest at mixing times near $1/2J$. Hence, the experiment loses sensitivity because of T_2 for long mixing times and because of insufficient buildup of magnetization at short mixing times.

One practical, sensitive alternative to shift correlation and selective proton decoupling experiments is to observe ^{1}H and decouple ^{15}N. In favorable cases, with samples in ^2H_2O, the otherwise unsplit resonances of His C2 and C4 and Trp C2 protons can be located, even though the coupling constant is only ~12 Hz. This coupling is much too small to permit the use of two-dimensional shift correlation experiments.

The chemical shift range for the peptide nitrogens is rather narrow, and the probability of obtaining a large number of detailed assignments in this region is not as high as for side chain nitrogens. However, many amide protons exchange only slowly, and spin coupling information is retained. Therefore, decoupling or shift correlation experiments linking the nitrogen atom to its proton used in conjunction with homonuclear COSY experiments can provide firm assignments.

Selective NOE's may provide useful information. However, especially for large proteins, spin diffusion among the protons may lead to spurious results. In some cases, it may be preferable to sacrifice sensitivity and resolution in the ^{15}N spectrum by working at lower field in order to decrease the effects of spin diffusion.

As expected, we have found that the ^{15}N resonances of histidine and the amino terminus are strongly pH dependent. In the spectrum of cytochrome c_2, we have also observed that the shifts of some of the ϵ-amino groups of lysine are pH dependent. Some of the groups exhibit anomalously low pK_a values and therefore change chemical shift at pH values near 8. The pK values are obtained by fitting to the Hill equation (Markley, 1975) by the same procedure as is used in proton NMR.

From the results we have obtained, it is clear that directly observed ^{15}N NMR spectroscopy of uniformly enriched proteins is a practical technique for the study of protein structure and dynamics. To obtain detailed information, it is usually necessary to observe single-site resonances. Hence, the tech-

nique will be most useful in cases in which the residues of importance are rare in the sequence or whose resonances are resolved from those of like residues because of protonation, metal ligation, hydrogen bonding, or other interactions that lead to unusual chemical shifts.

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Registry No. RuBPCase, 9027-23-0; cyt c_2, 9035-43-2; cyt c', 9035-41-0; NH_4^+, 14798-03-9; ^{15}N, 14390-96-6.

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Triplet-State Detection of Labeled Proteins Using Fluorescence Recovery Spectroscopy

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ABSTRACT: The experimental procedures for detecting the triplet states of chromophores in solutions (cuvettes) by fluorescence recovery spectroscopy (FRS) are described in detail, together with applications in studies of protein structure and protein–cell interactions in the microsecond to millisecond time domain. The experimental configuration has been characterized by measuring the emission intensities and anisotropies of eosin and erythrosin immobilized in poly(methyl methacrylate). The fluorescence data are compared with those from phosphorescence emission measurements and with theoretical predictions. Triplet-state lifetimes were obtained in 5 mM phosphate buffer, pH 7.0, of concanavalin A labeled with eosin, tetramethylrhodamine, and fluorescein and of α_2-macroglobulin labeled with the first two probes. In the case of labeled concanavalin A, iodide quenching measurements gave bimolecular rate constants of approximately 10^9 M^{-1} s^{-1}. The usefulness of FRS for studying protein–cell interactions is exemplified with eosin-labeled concanavalin A bound to living A-431 human epidermoid carcinoma cells. Finally, the advantages and disadvantages of the technique are compared to those of the alternative phosphorescence emission method.

The fluorescence properties of small molecules are of great utility in the elucidation of macromolecular dynamics and structure (Rigler & Ehrenberg, 1976). This has been particularly true over the past two decades, in which developments in computer and laser technology have enhanced experimental capabilities. More recently, the excited triplet states of appropriate probe molecules have been exploited since their emission processes occur over time scales comparable to protein rotation in larger complexes and in membranes (Cherry, 1978;

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